PTL.2020 G (1)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 June 2002 (27.06.2002)

PCT

(10) International Publication Number
WO 02/50296 A1

(51) International Patent Classification[7]: C12P 7/18, C12N 1/20

(21) International Application Number: PCT/FI01/01127

(22) International Filing Date:
19 December 2001 (19.12.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
20002792 20 December 2000 (20.12.2000) FI

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/50296 A1

(54) Title: PROCESS FOR PRODUCING D-MANNITOL

(57) Abstract: High concentration of free cells of heterofermentative lactic acid bacteria (LAB) in a resting or slowly growing state are used to convert fructose into mannitol. Efficient volumetric mannitol productivities and mannitol yields from fructose are achieved in a process applying cell-recycle, continuous stirred tank reactor and/or circulation techniques with native LAB cells or with LAB cells with inactivated fructokinase gene(s). Mannitol is recovered in high yield and purity with the aid of evaporation and cooling crystallization.

Process for Producing D-mannitol

Field of the Invention

This invention relates to the use of microorganisms, namely lactic acid bacteria (LAB), and concerns particularly a new process for the bioconversion of fructose into mannitol with free, native or fructokinase inactivated cells in a resting or a slowly growing state. The invention also relates to the re-use of the cell biomass for successive bioconversions.

Background of the Invention

D-mannitol is a six-carbon sugar alcohol, which is about half as sweet as sucrose. It is found in small quantities in most fruits and vegetables (Ikawa *et al.*, 1972; Bär, 1985). Mannitol is widely used in various industrial applications. The largest application of mannitol is as a food additive (E421), where it is used e.g. as a sweet tasting bodying and texturing agent (Soetaert *et al.*, 1999). Crystalline mannitol is non-sticky, i.e. it prevents moisture absorption, and is therefore useful as coating material of e.g. chewing gums and pharmaceuticals. In medicine, mannitol is used as osmotic diuretic for intoxication therapy and in surgery, parenteral mannitol solutions are applied to prevent kidney failure (Soetaert *et al.*, 1999). Mannitol is also used in brain surgery to reduce cerebral edema.

At present, commercial production of mannitol is done by catalytic hydrogenation of invert sugar with the co-production of another sugar alcohol, sorbitol. Typically, the hydrogenation of a 50/50-fructose/glucose mixture results in a 30/70 mixture of mannitol and sorbitol (Soetaert *et al.*, 1999). Besides the fact that mannitol is the by-product of the chemical production process and thus liable to supply problems, it is also relatively difficult to separate from sorbitol. In contrast to most sugars and other sugar alcohols mannitol dissolves poorly in water (13% (w/w) at 14°C (Perry *et al.*, 1997)). Cooling crystallization is therefore commonly used as a separation method for mannitol. However, according to Takemura *et al.* (1978) the yield of crystalline mannitol in the chemical process is still only approximately 17% (w/w) based on the initial sugar substrates.

In order to improve the total yield of mannitol it would be advantageous to develop a process with mannitol as the main product and with no sorbitol formation. Some alternative processes based on the use of microbes have been suggested in the literature. Yeast, fungi, and LAB especially, are able to effectively produce mannitol without co-formation of sorbitol (Itoh *et al.* 1992). Among LAB only heterofermentative species are known to convert fructose into mannitol (Pilone *et al.* 1991; Axelsson, 1993; Soetaert *et al.* 1999). Species belonging to the genera *Leuconostoc*, *Oenococcus* and *Lactobacillus* particularly, have been reported to produce mannitol effectively. In addition to mannitol these microbes co-produce lactic and acetic acid, carbon dioxide and ethanol. These by-products are, however, easily separable from mannitol.

Soetaert and co-workers have studied the bioconversion of fructose into mannitol with free cells of *Leuconostoc pseudomesenteroides* ATCC-12291 (Soetaert *et al.*, 1994). Using a fed-batch cultivation protocol they reached a maximum volumetric productivity of 11 g mannitol/L/h and a conversion efficiency of approximately 94 mole-%. Recently, Korakli *et al.* (2000) reported a 100% conversion efficiency with *Lactobacillus sanfranciscensis* LTH-2590. Other heterofermentative LAB reported to be good producers of mannitol include *Leuconostoc mesenteroides*, *Oenococcus oeni*, *Lactobacillus brevis*, *Lactobacillus buchneri* and *Lactobacillus fermentum* (Pimentel *et al.*, 1994; Salou *et al.* 1994; Erten, 1998; Soetaert *et al.* 1999).

In JP62239995, Hideyuki *et al.* (1987) used free cells of *Lb. brevis*. The volumetric mannitol productivity achieved in batch fermentation was 2.4 g/L/h.

EP0486024 and EP0683152 describe a strain named *Lb.* sp. B001 with volumetric mannitol productivities of 6.4 g/L/h in a free cell batch fermentation (Itoh *et al.*, 1992; Itoh *et al.*, 1995).

More recently, Ojamo *et al.* (2000) have submitted a patent application for a process for the production of mannitol by immobilized LAB. In this process the average volumetric mannitol productivity and conversion efficiency achieved were approximately 20 g/L/h and 85%, respectively. A low-nutrient medium was used which considerably lowers the production costs. Immobilization also enables the re-use of cell biomass for successive batch fermentations.

These inventions have not yet replaced the conventional hydrogenation process. The free cell bioconversion processes described to date are not entirely suitable for industrial scale production. Volumetric productivities in the range of 20 g/L/h, as achieved with the immobilization process, should however, be adequate for profitable production. In order to further develop the features of the bioconversion alternative, factors such as equipment investment costs, robustness of the process, medium composition (raw material costs), and mannitol yields must be considered and improved. The goal of the present invention is to overcome the prior disadvantages, such as the low productivities obtained with the free cell bioconversion systems and the low mannitol yields characteristic for all available bioconversion systems. Thus, the goal of the present invention is to develop a bioconversion process, which is feasible both technically and economically.

Summary of the Invention

The present invention is accomplished to overcome the disadvantages mentioned above. The present invention provides a process in which a high concentration of free cells of lactic acid bacteria is applied to the bioconversion of fructose into mannitol. During the bioconversion phase the cells are kept in a resting or a slowly growing state by supplementing to the fructose containing solution only minimal amounts of complex nutrients required for growth. The present invention describes the use of an efficient, high-yield mannitol-producing strain in the process. The strain in question was identified by comparing the mannitol production capabilities of different LAB species kept in a resting or slowly growing state. The present invention also provides an efficient, robust production process with productivities over 20 g mannitol/L/h. In addition, the process concept described here is simple to apply in industrial scale, and because of the low-nutrient medium used in it, the raw material costs are minimized. Furthermore, by inactivating the fructokinase gene a 100% yield of mannitol from fructose is obtained.

The invention thus concerns a process for the production of mannitol by bioconversion, which process comprises the steps of bringing a high initial concentration of free, mannitol-producing lactic acid bacterial cells into contact with a low-nutrient medium supplemented with a substrate convertible into mannitol, and a cosubstrate, in a bioreactor system; performing the bioconversion under conditions suitable for converting said

substrate into mannitol; separating the bacterial cells from the medium by filtration to obtain a cell-free solution; recovering from the cell-free solution the mannitol produced; and reusing the separated bacterial cells in the bioreactor system.

Consequently, an object of the present invention is to provide a semi-continuous or a continuous process for the production of mannitol. One process alternative to accomplish this is the re-use of free cell biomass in successive batch bioconversions as shown in Fig. 1. When the initial fructose is depleted the cells are concentrated e.g. by tangential flow filtration (TFF), whereby the mannitol is removed from the bioconversion reactor in the cell-free permeate. The cell concentrate is then diluted with fresh fructose-rich solution and a new batch is started. During the bioconversion the cells are kept in a resting or slowly growing state.

Another embodiment of the present invention provides a process where a fructose-rich solution in a mixing reactor is circulated through a bioconversion reactor containing free cells in a resting or slowly growing state. The cells are kept in the bioconversion reactor by cell-recycle techniques (e.g. TFF; see Fig. 2) and the cell-free permeate is re-circulated back to the mixing reactor. The volume in the bioconversion and mixing reactors is kept approximately constant.

A third embodiment of the present invention is a continuous process where a fructose-rich solution is added to a bioconversion reactor containing free cells in a resting or slowly growing state. The cells are kept in the bioconversion reactor by cell-recycle techniques (e.g. TFF) and the mannitol-rich, cell-free permeate is directed to downstream processing via a recovery tank (Fig. 3). The volume of the bioconversion reactor is kept constant by continuous stirred tank reactor (CSTR) techniques (e.g. by level controller, calibrated feed and harvest pumps, or balancing the bioconversion reactor).

Furthermore, the present invention relates to the use of LAB in the process. Several species can be used in the process with varying yields and productivities (see Table 1 in Example 9). For instance, *Leuconostoc pseudomesenteroides* has a high productivity, but a yield less than 80%. This is due to a strong leakage of fructose substrate to the phospho-ketolase pathway via fructokinase-catalyzed phosphorylation. On the other hand, *Lactobacillus sanfranciscensis* gives a 100% mannitol yield from fructose, but is low in

productivity (less than 0.5 g/L/h). To have both a high productivity and to maximize the yield, the fructokinase gene(s) is/are inactivated in the present invention in a high productivity species like *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides* or *Lactobacillus fermentum*.

Consequently, further objects of the invention are bacterial strains of the genus *Lactobacillus* or *Leuconostoc*, in which the fructokinase enzyme(s) is/are inactivated.

Brief Description of the Drawings

Figure 1. Batch process alternative. *Phase 1*: Bioconversion. *Phase 2*: Product recovery and cell concentration with tangential flow filtration (TFF). *Phase 3*: Addition of fresh fructose-rich solution to the concentrated cell suspension.

Figure 2. Circulation process alternative. The cells are kept within the system consisting of the bioconversion reactor unit, the retentate side of the filtration unit and the circulation loop. Fructose-containing solution is pumped from the mixing reactor at the same flow rate as permeate is added to the mixing reactor.

Figure 3. Continuous process alternative. Fresh fructose-rich solution is prepared in the mixing reactor, which is then transferred into the feed tank. Solution is added to and removed from the bioconversion reactor system, consisting of the bioconversion reactor unit, the retentate side of the filtration unit and the circulation loop, at the same flow rates.

Detailed Description of the Invention

The primary embodiment of the present invention is a process in which mannitol is produced by bioconversion from fructose with the aid of free native or fructokinase inactivated LAB cells kept in a resting or a slowly growing state. The volumetric mannitol productivities and mannitol yields from fructose for such a system are preferably above 10 g/L/h and 90 mole-%, respectively.

The preferred substrate for the bioconversion is fructose. Sucrose can be used as well. In addition, glucose is preferred as a co-substrate for the production of NAD(P)H, which is

needed as a cofactor in the bioconversion of fructose into mannitol. Based on a 100 mole-% bioconversion yield of fructose into mannitol, the preferred molar ratio of fructose and glucose is 2:1. Typically among heterofermentative lactic acid bacteria a varying fraction of fructose that has been transported into the cells is phosphorylated by fructokinase-catalysis to form fructose-6-P and thus, channeled into the phosphoketolase pathway. The "leaking" fructose carbon skeleton is then converted stepwise into end products such as acetic and lactic acid, ethanol and carbon dioxide. When fructose is leaking to the phosphoketolase pathway and when the mannitol yield from fructose is less than 100 mole-%, it is preferable to increase the fructose to glucose ratio to avoid residual glucose concentrations. Preferred initial concentrations of fructose and glucose vary from 50 to 200 g/L and 20 to 100 g/L, respectively. The upper limit of initial fructose concentration is usually set by the maximum solubility of mannitol at the bioconversion conditions in question. An end concentration of mannitol over the maximum solubility would result in crystalline mannitol to form in the bioconversion reactor, which preferably should be avoided.

Instead of using high-purity fructose and/or glucose as the substrates, also respective compounds with a lower purity can be used as the substrate for the cells. This is preferred in order to lower the raw material costs, which are strongly influenced by the price of fructose and glucose. Besides the sugars noted earlier, the bioconversion medium also needs to be supplemented at least with complex nitrogen sources, magnesium and manganese ions. The preferred complex nitrogen sources are yeast extract, preferably in initial concentrations of 0.1 to 1 g/L, and tryptone, preferably in initial concentrations of 0.2 to 2 g/L. The concentrations of magnesium and manganese ions are preferably in the range from 0.1 to 0.5 g/L and 0.01 to 0.1 g/L, respectively. Concentrations providing optimum mannitol production depend on the strain in question and can therefore, deviate from the numbers shown above. The magnesium and manganese ions can preferably be added in the form of respective sulphates. Alternative and less expensive complex nitrogen sources are e.g. soybean and cottonseed meal, corn steep liquor (CSL), yeast hydrolysates etc.

The preferred minimum concentration of free cells in the bioconversion reactor is 5 g dry cell weight/L. A value over 10 g/L is preferred. The initial cell biomass production, which enables the first bioconversion cycle to proceed, can be achieved by cultivating the cells

in a nutrient-rich growth medium, applying techniques such as batch, fed-batch, or CSTR cell-recycling. The cells are then concentrated to high cell densities, preferably 25 to 100 g dry cell weight/L, by e.g. tangential flow filtration (TFF) or centrifugation. Once the cells are in the bioconversion reactor, in the preferred concentrations mentioned above, the same cells can be used for several successive batch bioconversions (see Figures 1 and 2). Hence, the processes according to alternatives shown in Figures 1 and 2 of the present invention are semi-continuous.

The bioconversion and the mixing reactors are preferably agitated vessels with the possibility to measure and control on-line the temperature and pH of the bioconversion medium. Pressure indicators should preferably also be available. The carbon dioxide formed during the bioconversion is preferably released via the headspace either in the bioconversion or in the mixing reactor or both. The vessels are preferably made out of food-grade stainless steel material and the system should preferably be suitable for aseptic process protocols. Several reactors may be used in series and/or in parallel. For instance, nitrogen flushing of the media can be used to improve the mannitol yields from fructose and the CO_2 removal from the bioreactors.

The temperature and pH of the bioconversion medium should preferably be controlled either in both the bioconversion and the mixing reactor or only in one of the reactors. The temperature can be adjusted either with e.g. water or steam, whereas the pH can be adjusted with e.g. NaOH, KOH, NH_4OH, HCl or H_2SO_4 solutions. The temperature and pH should preferably be adjusted within the respective optimum values in order to provide maximum mannitol productivity.

A suitable microorganism, in its native form, should preferably express mannitol dehydrogenase activity and produce mannitol as its main metabolite. Among suitable microorganisms are *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus fermentum*, *Lactobacillus sanfranciscensis*, and *Oenococcus oeni*. The preferred species is *Leuconostoc mesenteroides* and especially strain ATCC-9135. The present invention is, however, not limited to these microorganisms. The present invention also refers to microorganisms with activities similar to those mentioned above. Also microorganisms derived, by e.g.

recombinant techniques, from microorganisms mentioned above or from microorganisms with activities similar to those mentioned above, may be used in the process.

If the concentration of free cells in the bioconversion reactor is increasing too much so that e.g. the productivity is decreasing from a normal value, a suitable volume of the cell suspension can be removed from the system. In the batch version of the present invention (Fig. 1) this is preferably done before the fresh bioconversion medium is added to the high cell density suspension, in order to start a new batch-cycle. In the circulation version of the present invention (Fig. 2) the removal is preferably done while the mixing vessel is emptied after fructose depletion and then refilled with fresh bioconversion medium. In the continuous version of the present invention (Fig. 3) e.g. the dilution rate and the contents of the feeding solution are used to control the production of mannitol. If it is necessary to remove cells from the continuous bioconversion reactor, it can be done applying e.g. TFF techniques.

While a microfiltration membrane or a large ultrafiltration membrane (e.g. 1000 kDa) is used in the TFF equipment for cell separation, it is not expected that any other component would be concentrated to harmful levels in the system, while these are most likely removed from the bioconversion reactor with the permeate or alternatively consumed by the cells.

The inactivation of the fructokinase activity is accomplished either by classical mutagenesis or by targeted gene inactivation techniques. Classical mutagenesis is done by treating growing cells of LAB with 1-methyl-3-nitro-1-nitrosoguanidine and selecting for bacteria, which cannot grow on fructose as the sole carbon source. The obtained mutants are further tested for their ability to import fructose into the cell to assure that the growth defect on fructose is not caused by a mutation present in fructose permease. The fructose transport is verified using radioactively labeled fructose in the growth medium and detecting the radioactivity in separated washed cells. Alternatively, the transport of fructose can be indirectly confirmed by measuring the conversion of fructose to mannitol in growth medium containing fructose.

The targeted inactivation of the fructokinase gene is done either by disrupting or by deleting the fructokinase gene. The inactivation plasmids for both purposes are

constructed using a vector plasmid with temperature sensitive replication origin to enhance the integration event to the bacterial chromosome. One example of this kind of plasmid is pGhost4, which is a wide host-range plasmid, capable of replicating in many Gram-positive bacteria (Biswas *et al.*, 1993). In the first phase the inactivation plasmid is transferred to LAB by electroporation and transformants are selected at a permissive temperature using antibiotic selection. In the second phase, integration of the plasmid to the bacterial chromosome is achieved by growing the transformants at a non-permissive temperature to plasmid replication, using still antibiotic selection.

In the disruption construct an internal fragment of the fructokinase gene is cloned to the vector plasmid and integration at the fructokinase locus will interrupt the coding sequence and thus prevent the formation of an active fructokinase enzyme. In the case of targeted deletion of the fructokinase gene, integration of the deletion plasmid in the second phase does not disrupt the coding sequence, but creates two regions of homologous sequences, which serve as excision sites in later steps. These regions determining the excision sites are cloned in the deletion plasmid in a consecutive order and all DNA sequences between these regions will be deleted when homologous recombination occurs. Also all plasmid sequences, together with the antibiotic resistance gene, will be removed from the bacterial chromosome. After integration of the deletion plasmid the transformant bacteria are grown without antibiotic and clones sensitive to antibiotic are selected and tested for growth on fructose. The clones that cannot grow on fructose as sole carbon source are selected. The conversion of fructose to mannitol will be determined, and also the growth on the same substrates, used for native LAB strains, will be tested.

Mannitol is the main bioconversion product of the present invention. Other bioconversion products, which are dissolved in the medium, are e.g. acetic and lactic acid, and ethanol. Most of the carbon dioxide in the liquid medium is preferably removed from the system as gaseous carbon dioxide through agitation and/or nitrogen flushing of the medium. The liquid product solution is separated from the cells by TFF, as shown in Fig. 1 (no additional cell separation step is needed in the other two process alternatives of the present invention). The rest of the product recovery process comprises of the following unit operations: concentration, crystallization, separation, drying, and homogenization. Alternatively also other metabolites formed, besides mannitol, can be recovered from the bioconversion medium.

The concentration of the liquid product solution is preferably done by evaporation. The heated concentrate is then transferred to a cooling crystallization unit, where mannitol crystals fall out when the temperature of the solution is decreased. Next the crystals are separated from the mother liquor by a drum separator and the crystals thereby collected (crystals A). The mother liquor is either added to the next recovery cycle or re-crystallized separately (crystals B). Alternatively, the mother liquid, if containing residual fructose, can be recycled back to the bioconversion step. The crude crystals (A and B) are dissolved in hot water, where after the solution is re-crystallized in a cooling crystallization unit. After a second drum separation step the white crystals are dried in a vacuum or under-pressure oven. Finally, if needed, the dry crystals are homogenized by a suitable method. According to the protocol presented above the total mannitol recovery yield and crystal purity achieved, is preferably 50 to 100 mass-%, and 95 to 100 mass-%, respectively.

Example 1

Production of cells for the bioconversion phases 1

A bench-top bioreactor containing 9.7 L of nutrient-rich fermentation medium (Soetaert *et al.*, 1999) was inoculated with 300 mL of a 16-h cell culture of *Leuconostoc pseudomesenteroides* ATCC-12291 grown in an inoculation medium (Soetaert *et al.*, 1999). The temperature of the growth medium (10 L) was set first at 20°C and after 56 h raised to 25°C. The pH was controlled at 5.0. The solution was slowly agitated.

After about 66 hours the cultivation was stopped and the cells recovered by tangential flow filtration (Pellicon® 2 Mini Holder and Biomax® 1000 (V screen) membrane, Millipore Corp., USA). From an initial volume of 10.9 L (~3 g dry cell weight/L) a 0.7-L cell concentrate (~47 g dry cell weight/L) was obtained by this filtration technique. The cell-free permeate (10.2 L) could thereafter be used for study of mannitol recovery. The cell concentrate can be used as the initial biomass for the processes described in Examples 3–5.

The volumetric mannitol productivity of this free cell process was 1.7 g/L/h.

Example 2

Production of cells for the bioconversion phases 2

A bench-top bioreactor containing 1.9 L of MRS growth medium (40 g/L glucose) was inoculated with 100 mL of a 10-h cell culture of *Leuconostoc mesenteroides* ATCC-9135 also grown in a MRS growth medium (30 g/L glucose). The temperature and pH of the growth medium (2 L) were set at 30°C and 6.0, respectively. The solution was slowly agitated.

About 9.5 hours later the cells were harvested by centrifugation. The cell pellet was then suspended in a fresh bioconversion medium (See Examples 3–5).

Example 3

Production of mannitol by bioconversion in a batch mode (Fig. 1)

The cell pellets obtained in Example 2 was suspended with fresh bioconversion medium and transferred aseptically into a bioconversion reactor. The total volume of the solution was 425 mL and it had the following initial composition: 100 g/L fructose, 50 g/L glucose, 1 g/L tryptone, 0.5 g/L yeast extract, 2.62 g/L $K_2HPO_4 \cdot 3H_2O$, 0.2 g/L $MgSO_4$, and 0.01 g/L $MnSO_4$. The cell concentration during the bioconversion was approximately 10 g dry cell weight/L.

The temperature control was set at 30°C and the pH was controlled at 5.0 with 3 M NaOH. The solution was slowly agitated.

After 4.5 hours of bioconversion time the cells had consumed all of the sugars and the experiment was ended. The average volumetric mannitol productivity for the process was 20.7 g/L/h. The mannitol yield from fructose was 91.2 mole-%.

Furthermore, the product solution and cells can be separated by e.g. TFF, and the cell concentrate re-used in successive batch bioconversions according to the process description in Fig. 1.

Example 4

Production of mannitol by bioconversion with circulation (Fig. 2)

The experiment set up is shown in Fig. 2. The cell pellets, obtained as described in Example 2, were suspended in fresh bioconversion medium lacking the sugars and transferred aseptically to the bioconversion reactor unit. The volume in the bioconversion reactor unit was 0.4 L. A TFF unit (Pellicon® 2 Mini Holder and Biomax® 1000 (V screen) membrane, Millipore Corp., USA) was attached to the bioconversion bioreactor unit and the permeate flow was lead to a mixing reactor. The mixing reactor (volume 1.0 L) was standing on a balance and the mass of the reactor was kept constant by circulating medium back to the bioconversion reactor unit. The total volume of the whole system was 1.5 L and the medium had the following initial composition: 100 g/L fructose, 50 g/L glucose, 1 g/L tryptone, 0.5 g/L yeast extract, 2.62 g/L $K_2HPO_4 \cdot 3H_2O$, 0.2 g/L $MgSO_4$, and 0.01 g/L $MnSO_4$. The cell concentration in the bioconversion reactor was 8.7 g dry cell weight/L.

The temperature and the pH were controlled both in the bioconversion reactor and in the mixing reactor units. The temperature control was set at 30°C and the pH was controlled at 5.0 with 3 M NaOH. Mixing was applied in both reactors.

After 9 hours of bioconversion time the cells had consumed all of the sugars and the experiment was ended. The average volumetric mannitol productivity for the process was 21.6 g/L/h. The mannitol yield from fructose was 94.0 mole-%.

Example 5

Production of mannitol by bioconversion in a continuous reactor (Fig. 3)

The experiment set up is shown in Fig. 3. The cell pellets, obtained as described in Example 2, were suspended in fresh bioconversion medium lacking the sugars and transferred aseptically to the bioconversion reactor unit. A TFF unit (Pellicon® 2 Mini Holder and Biomax® 1000 (V screen) membrane, Millipore Corp., USA) was attached to the bioconversion bioreactor unit and the permeate flow was lead to a recovery tank. The total volume in the bioconversion reactor unit, retentate side of the filtration unit, and in

the circulation loop was 1.0 L. The bioconversion reactor unit was standing on a balance and the mass of the reactor was kept constant by adding fresh medium from a feed tank. The feeding solution following initial composition: 25 g/L fructose, 12.5 g/L glucose, 1 g/L tryptone, 0.5 g/L yeast extract, 2.62 g/L $K_2HPO_4 \cdot 3H_2O$, 0.2 g/L $MgSO_4$, and 0.01 g/L $MnSO_4$. The cell concentration in the bioconversion reactor, at dilution rate 0.68 1/h, was approximately 6.9 g dry cell weight/L.

The temperature control was set at 30°C and the pH was controlled at 5.0 with 3 M NaOH. The reactor was slowly agitated. A volumetric mannitol productivity of 12.5 g/L/h was achieved. The mannitol yield from fructose was 93.0 mole-%.

Example 6

Inactivation of the gene encoding fructokinase by random mutagenesis

Chemical mutagenesis of *L. pseudomesenteroides* ATCC-12291 was done using log-phase cells (OD_{600} 1.0) grown in M17 supplemented with 1% glucose (GM17). Cells washed with 50 mM sodium phosphate buffer, pH 7, were treated with 1-methyl-3-nitro-1-nitrosoguanidine, 0.5 mg/ml, for 40–50 min, at room temperature, and washed three times with the buffer above. Washed cells were incubated in GM17, for 1 hour, at 30°C, and plated on GM17 agarose, incubated 2 days at 30°C. Colonies on GM17 plates were replica-plated on a chemically defined medium (CDM; Anon., 2000) supplemented with either 1% glucose or 1% fructose. After 2 days of incubation at 30°C colonies growing on glucose, but not on fructose, were selected. Conversion of fructose to mannitol will indicate that the fructose permease is not affected by the mutagen. The fructokinase inactivated production strain, which was able to convert fructose to mannitol, was named BPT-143. The strain was deposited according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Mascheroder Weg 1b, D-34124 Braunschweig, Germany on 13 November 2001 with the accession number DSM 14613.

Example 7

Inactivation of the gene encoding fructokinase by directed mutagenesis

Inactivation plasmid for disrupting the fructokinase gene(s) of *Lb. fermentum* is constructed by joining an internal fragment of a fructokinase gene between suitable restriction sites of pGhost4. The ligation mixture is electroporated to *Lactococcus lactis*, transformants are incubated for 1 day, at permissive temperature, 30°C, using erythromycin (Em, 5 µg/ml) and screened by PCR with pGhost4-specific primers. Recombinant plasmids, containing the internal fragment of fructokinase gene, are isolated and electroporated to *Lb. fermentum*. Transformants are incubated anaerobically, for 1 day, at 30°C, and verified by PCR with the previously mentioned primers. Clones carrying the recombinant plasmids selected for the integration experiments are grown over night, at 30°C, in MRS growth medium supplemented with 5 µg/ml Em. These cell suspensions are used as inoculate for new cultures grown for 5 hours at 42°C in same medium. Then the cell suspensions are diluted 1:100 000, plated on MRS-Em, and incubated for 2 days at 42°C. Colonies arising in the presence of Em at 42°C will have a disruption plasmid integrated to the chromosome at the fructokinase locus. Disruption of the fructokinase gene(s) will result in reduced fructokinase activity of the disruption transformants compared to the wild type *Lb. fermentum* grown in MRS or CDM supplemented with different sugars (sucrose, fructose, lactulose, maltose, galactose or ribose) and 5 µg/ml Em. Disruption of the fructokinase gene(s) is confirmed by Southern blotting of the chromosomal DNA isolated from the clones with reduced fructokinase activity.

Fructokinase genes are deleted using the following protocol. Two 0.5 kb fragments amplified by PCR from *Lb. fermentum* chromosome, surrounding the targeted deletion site, are ligated to pGhost4. The ligation mixture is electroporated to *L. lactis*, transformants are incubated for 1 day at permissive temperature, 30°C, using erythromycin (Em, 5 µg/ml) and screened by PCR with pGhost4-specific primers. Plasmids containing the cloned fragments are isolated and electroporated to *Lb. fermentum*. Transformants are incubated anaerobically on MRS-Em plates for 1 day at 30°C and resulting colonies are verified by pGhost4-specific primers to ensure the presence of the recombinant plasmids and correct insert sizes. Raising the temperature as described for

the disruption plasmids will result in integration of the recombinant plasmid to the chromosome. Sites of the integration are confirmed by Southern blotting of chromosomal DNA isolated from the integrant strains. The *Lb. fermentum* carrying an integrated recombinant plasmid at a fructokinase locus is then grown without Em, at 42°C, for 100 generations and plated on MRS without Em. Omission of the antibiotic will result in dissociation of the integrated plasmid from the chromosome. Depending on the recombination site either restoration of the wild type or deletion of a fructokinase gene will happen. In both cases all foreign DNA will be removed from the chromosome. Em-sensitive clones are detected after replica plating on MRS with and without Em. Among the Em-sensitive clones those with reduced fructokinase activity are selected. Deletion of the fructokinase gene is confirmed by Southern blotting the chromosomal DNA isolated from the deletion strains.

Example 8

Production of mannitol by L. pseudomesenteroides with inactivated fructokinase gene (random mutagenesis)

L. pseudomesenteroides ATCC-12291 and the clone DSM 14613 (BPT 143) with inactivated fructokinase gene (see Example 6) were tested for mannitol production in parallel experiments. The growth medium had the following composition: 20 g/L fructose, 10 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 2.62 g/L $K_2HPO_4 \cdot 3H_2O$, 0.4 g/L $MgSO_4$, and 0.02 g/L $MnSO_4$. The temperature and pH was set at 30°C and 5.0, respectively. The bioconversion time was 8 hours. The mannitol yields from fructose for the native strain and the clone were 73.7 mole-% and 85.7 mole-%, respectively. Also, a 25% improvement in volumetric mannitol productivity was observed.

Example 9

Comparison of mannitol production capacity of lactic acid bacteria in a resting or slow-growing state

Pre-cultures of three of the most promising strains (preliminary comparison studies not shown) were grown in MRS growth medium. The cell suspensions were centrifuged and the cell pellets washed in 0.2 M phosphate buffer (pH 5.8). After an additional

centrifugation separation the cell pellets were resuspended in the same buffer. The concentrated cell suspensions (50 mL per strain) were added to bioreactors containing 450 mL of a bioconversion medium. After addition the composition of the solution was the following: 20 g/L fructose, 10 g/L glucose, 0.5 g/L tryptone, 0.25 g/L yeast extract, 2.62 g/L $K_2HPO_4 \cdot 3H_2O$, 0.2 g/L $MgSO_4$, and 0.01 g/L $MnSO_4$.

The temperature and pH of the bioconversion medium were set at 30°C and 5.0, respectively. The bioconversion media were slowly agitated. The key results are shown in Table 1.

Table 1. The volumetric mannitol productivities (r_{mtol}) and mannitol yields from glucose ($Y_{mtol/fru}$) after 8 hours of bioconversion time.

Strain:	r_{mtol} (g/L/h)	$Y_{mtol/fru}$ (mole/mole)
Leuconostoc mesenteroides ATCC-9135	2.3	97.8
Leuconostoc pseudomesenteroides ATCC-12291	1.5	79.6
Lactobacillus fermentum NRRL-1932	1.0	86.1

Example 10

Recovery of mannitol

The cell-free permeate, described in Example 1, was concentrated to approximately 250 g mannitol/L by evaporating with a Rotavapor unit. The concentrate (T = 35°C) was transferred into a cooling crystallization unit and the temperature was linearly (15 h) decreased to 5°C. The solution was slowly agitated. The crystals were separated by filtration and the mother liquor was re-crystallized as described above.

The wet crystals from the first and the second cycle were combined and dissolved in distilled water (T = 45°C). The mannitol concentration of the solution was approximately 300 g/L. The solution was transferred into a cooling crystallization unit and the

temperature was linearly (15 h) decreased to 5°C. The crystals were separated by filtration and finally, the wet crystals were dried overnight at 60°C.

The recovery yield was about 55 mass-% and the purity above 99.5 mass-%. The mannitol found in the washing solution gained from the last crystallization step can be re-used as part of the washing solution in the next recovery cycle. Adding this hypothetical amount of mannitol to the crystals obtained in the first recovery cycle a final recovery yield of about 71% was achieved.

Deposited microorganisms

The following microorganism was deposited according to the Budapest Treaty at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Mascheroder Weg 1b, D-34124 Braunschweig, Germany.

Microorganism	Accession number	Deposit date
Leuconostoc pseudo-mesenteroides BPT-143	DSM 14613	13 November 2001

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Claims

1. A process for the production of mannitol by bioconversion, comprising the steps of

- bringing a high initial concentration of free, mannitol-producing lactic acid bacterial cells into contact with a low-nutrient medium supplemented with a substrate convertible into mannitol, and a cosubstrate, in a bioreactor system,
- performing the bioconversion under conditions suitable for converting said substrate into mannitol,
- separating the bacterial cells from the medium by filtration to obtain a cell-free solution,
- recovering from the cell-free solution the mannitol produced, and
- reusing the separated bacterial cells in the bioreactor system.

2. The process according to claim 1, wherein the bacterial cells are native lactic acid bacterial cells.

3. The process according to claim 2, wherein the bacterial cells are of the strain *Leuconostoc mesenteroides* ATCC-9135.

4. The process according to claim 1, wherein the bacterial cells are fructokinase-inactivated lactic acid bacterial cells.

5. The process according to claim 4, wherein the cells are of the strain *Leuconostoc pseudomesenteroides* BPT143 (DSM 14613).

6. The process according to claim 1, wherein the substrate is fructose.

7. The process according to claim 1, wherein the cosubstrate is glucose.

8. The process according to claim 1, wherein the bioconversion is performed until at least 70%, preferably 90% or more of the said substrate has been consumed.

9. The process according to claim 1, wherein an average volumetric mannitol productivity of at least 10 g/L/h is achieved.

10. The process according to claim 1, wherein the bioconversion is performed as a batch process in a bioreactor system comprising a bioconversion reactor unit and a filtration unit.

11. The process according to claim 10, comprising the steps of separating the bacterial cells from the medium after the bioconversion step, and reusing said cells in successive bioconversions.

12. The process according to claim 1, wherein the bioconversion is performed as a circulation process in a bioreactor system comprising a bioconversion reactor unit, a filtration unit and a mixing reactor unit.

13. The process according to claim 12, wherein the bacterial cells are circulated between the bioconversion reactor unit and the filtration unit.

14. The process according to claim 12 or 13, comprising the steps of leading the cell-free solution obtained by the filtration into the mixing reactor, and transferring the solution from the mixing reactor back to the bioconversion reactor unit.

15. The process according to any one of the claims 12 to 14, comprising emptying the mixing reactor after the bioconversion step, and re-filling it with said low-nutrient medium supplemented with said substrate and co-substrate, to run successive bioconversions reusing said bacterial cells.

16. The process according to claim 1, wherein the bioconversion is performed as a continuous process in a bioreactor system comprising a mixing tank, a feed tank, a bioconversion reactor unit, a filtration unit and a recovery tank.

17. The process according to claim 16, wherein the bacterial cells are circulated between the bioconversion reactor unit and the filtration unit.

18. The process according to claim 16 or 17, comprising the steps of feeding the bioconversion reactor unit continuously with said low-nutrient medium supplemented

with said substrate and co-substrate, and removing the cell-free solution gained by filtration from the bioreactor to withhold a constant volume in the bioconversion reactor unit.

19. The process according to any one of the claims 10 to 18, wherein the filtration unit is a tangential flow filtration unit.

20. The process according to any one of the claims 10 to 18, wherein said bioconversion is run in series or in parallel.

21. A bacterial strain of the genus *Lactobacillus* or *Leuconostoc*, in which the fructokinase enzyme(s) is/are inactivated.

22. The bacterial strain according to claim 21, in which the fructokinase enzyme(s) is/are inactivated by random mutagenesis.

23. The bacterial strain according to claim 22, which is *Leuconostoc pseudomesenteroides* BPT143 (DSM 14613).

24. The bacterial strain according to claim 21, in which the fructokinase enzyme(s) is/are inactivated by directed mutagenesis.

25. Use of a bacterial strain of the genus *Lactobacillus* or *Leuconostoc*, in which the fructokinase enzyme(s) is/are inactivated, for producing mannitol.

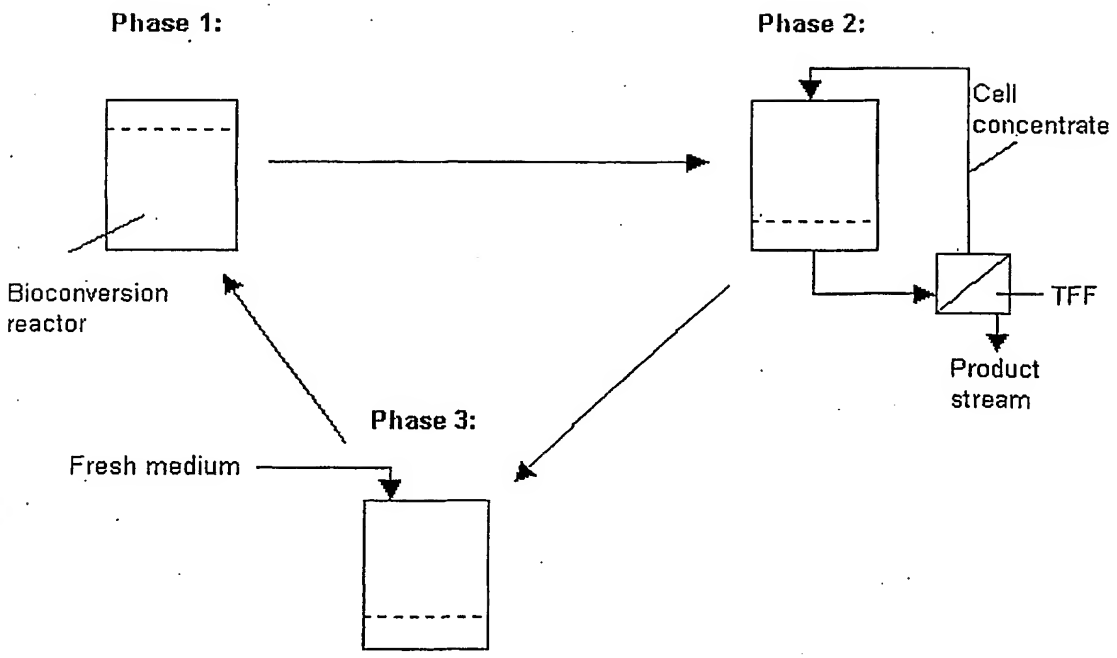
Phase 1:
Phase 2:
Cell concentrate
Bioconversion reactor
TFF
Product stream
Phase 3:
Fresh medium

FIGURE 1.

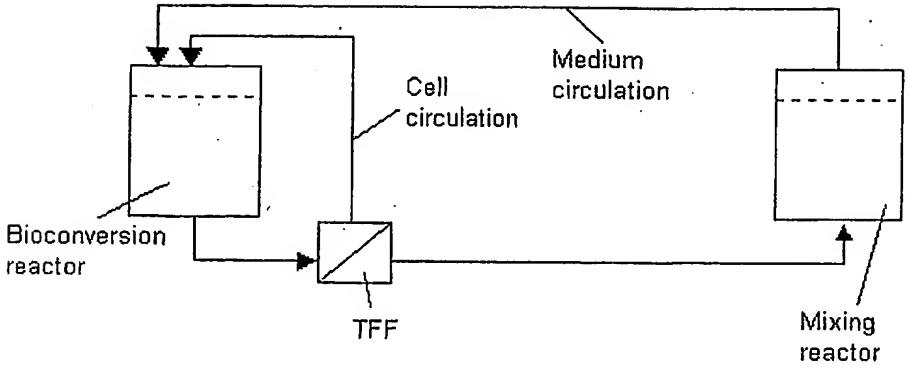
Medium
circulation
Cell
circulation
Bioconversion
reactor
TFF
Mixing
reactor

FIGURE 2.

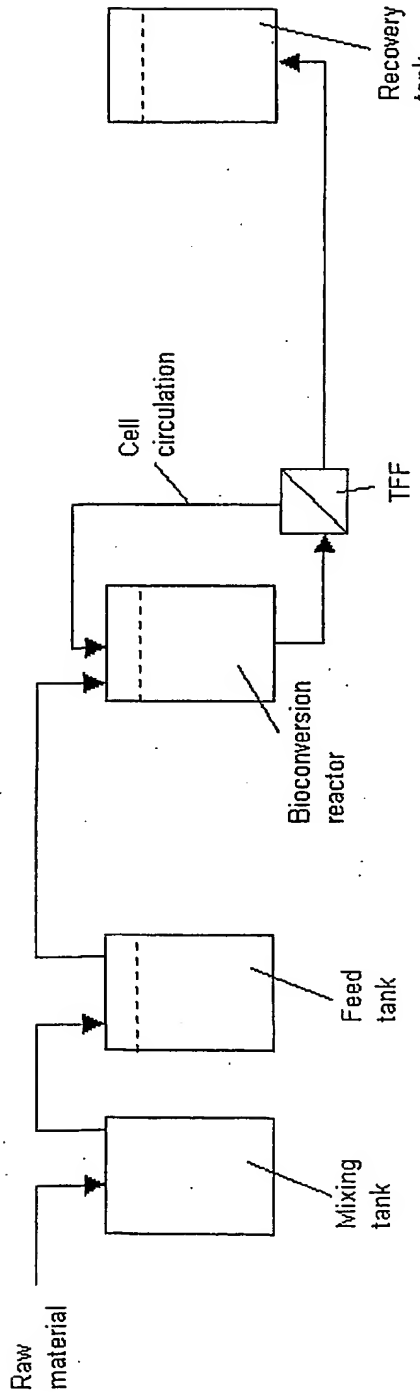
Recovery tank
Cell circulation
TFF
Bioconversion reactor
Feed tank
Mixing tank
Raw material

FIGURE 3.

Applicant's or agent's file reference	International application No.
37224	PCT/FI01/01127

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 13, line 26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
13 November 2001	DSM 14613

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European patent or a patent in Canada, Iceland or Norway is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or the corresponding information concerning the patent in Canada, Iceland or Norway or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the Commissioner (in Canada) or the person requesting the sample (Rule 28(4) EPC and the corresponding regulations in Canada, Iceland and Norway).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Raija Villikka	Authorized officer

Applicant's or agent's file reference	International application No.
37224	PCT/FI01/01127

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

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Date of deposit	Accession Number
13 November 2001	DSM 14613

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

When designating Australia, in accordance with regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No. 71), samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
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Authorized officer	Authorized officer

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 01/01127

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12P 7/18, C12N 1/20
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, CHEM. ABS DATA, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No:
Y	WO 0004181 A1 (XYROFIN OY), 27 January 2000 (27.01.00), page 3 - page 8	1-20
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Y	BIOSIS, PREV200000115336, Volume 22, No 1-2, pages 1-4, Korakli M. et al: "Production of mannitol by Lactobacillus sanfranciscensis", Advances in Food Sciences, January 2000, abstract	1-20
	--	
A	BIOSIS, PREV199192111041, Volume 55, No 4, pages 1549-1552, Soetaert W. et al: "Production of mannitol with leuconostocmesenteroides", Mededelingen van den Faculteit Landbouwwetenschappen rijksuniversiteit gent", 1990, abstract	1-20
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2002	14 -05- 2002
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Terese Persson/EÖ Telephone No. +46 8 782 25 00

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INTER[redacted]IONAL SEARCH REPORT

International application No.
PCT/FI 01/01127

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Fermentation and Bioengineering, Volume 8 85, No 2, 1998, Jong Won Yun et al: "A Comperative Study of Mannitol Production by Two Lactic Acid Bacteria", pages 203-208	1-20

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INTERNATIONAL SEARCH REPORT
Information on patent family members

01/05/02

International application No.
PCT/FI 01/01127

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0004181 A1	27/01/00	AU 4912799 A	07/02/00
		EP 1097237 A	09/05/01
		FI 981615 D	00/00/00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI01/01127

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI01/01127

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

The present claim 1 relates to a method for producing mannitol comprising the steps of:
-bringing free mannitol-producing lactic acid bacterial cells into contact with a substrate convertible into mannitol and a co-substrate
-performing the convertion under conditions suitable for converting said substrate into mannitol
-separating the bacterial cells from the medium
-recovering the mannitol produced from the solution
-reusing the separated bacterial cells
The present claim 21 relates to a bacterial strain of the genus Lactobacillus or Leuconostoc in which the fructokinase enzyme(s) is/are inactivated.
In the method according to claim 1 can any lactic acid bacteria be used as long as it produces mannitol. The common feature between the method and the bacterial strain is that the method uses lactic acid bacterial cells and the strain is of the genus Lactobacillus or Leuconostoc (also lactic acid bacterial cells). According to Korakli et al., Adv. Food. Sci., Vol. 22, No. 1-2, pp. 1-4, 2000, is it known to use bacterial cells belonging to the genus of Lactobacillus for mannitol production. Consequently, there is no "special technical feature" to link the method to the bacterial strain. The application is consequently considered to lack unity and contains the following 2 inventions:

1) A method for producing mannitol using mannitol-producing lactic acid bacterial cells. Claims 1-20.
2) A bacterial strain of the genus Lactobacillus or Leuconostoc in which the fructokinase enzyme(s) is/are inactivated and use of the strain for producing mannitol. Claims 21-25.

Only the first of these inventions has been searched since no additional fees were paid.

PT 1.20204

(4)

2/2 - (C) BIOSIS / [redacted]OSIS
AN - PREV19878302[redacted]
TI - FRUCTOSE META[redacted]LISM IN ---ZYMOMONAS-------MOBILIS-[redacted]
AU - VIIKARI L; KORHOLA M
AUAF- VTT, BIOTECH. LAB., TIETOTIE 2, SF-02150 ESPOO, FINL.
PUB - APPLIED MICROBIOLOGY AND BIOTECHNOLOGY
- 1986
VOL - 24
NR - 6
PG - 471-476
AB - In the metabolism of fructose by Zymomonas, the ethanol yield is decreased due to the formation of dihydroxyacetone, mannitol and glycerol. The reduction of fructose to mannitol by an NADPH-dependent ---mannitol--- ---dehydrogenase--- is apparently coupled to the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase, which exhibits higher activity with NADP than with NAD as cofactor. The relatively low cell yield on fructose can partly be explained by the loss of ATP in the formation of dihydroxyacetone and glycerol and partly by the toxic effect of dihydroxyacetone and acetaldehyde on the growth of the organism.